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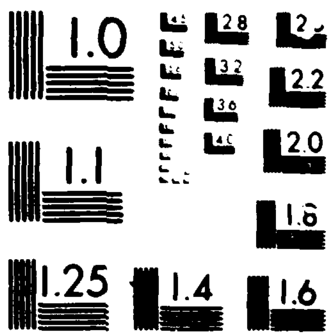
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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Immunoenhancing drugs were studied both for their general effects on host immunity and their effects on immune interactions with microbial infections. These studies focused preferentially on defined single molecules with immunomodulating properties, both wholly synthetic molecules (muramyl dipeptide was the prototype) and those produced by recombinant DNA techniques (gamma interferon was the prototype). Fungi were the microbes of interest. The effects of immunomodulators on fungicidal activity of tissue microphages from different anatomic compartments (pulmonary, peritoneal) were compared. We demonstrated that polymorphonuclear leukocytes (PMN) can be activated in vivo for enhanced fungicidal activity as a byproduct of an immunological or an inflammatory reaction, and that this could be reproduced with lymphokines or other immunomodulators. The mechanism of PMN activation was defined with respect to the oxidative mechanisms involved.

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Format of this Final Report: This report reviews the entire contract. However, as each of the first 3 years has already been detailed in annual reports previously submitted (see DTIC accession numbers AD-A135326, AD-A148592, and AD-A162997, respectively), these years will only be summarized briefly.

Year 1: Immunoenchancing drugs were studied both for their general effects on host immunity and their effects on immune interactions with microbial infections. These studies focused preferentially on defined single molecules with immunomodulating properties, and on fungi as the microbes of interest. The prototype immunomodulator studied was muramyl dipeptide (MDP). We showed that MDP in vivo enhanced cellularity and mitogen responses in some lymphoid compartments (lymph nodes) and depressed responses in others (spleen). The optimum dose and interval for effect were defined, and subpopulations of the compartments were fractionated which were responsible for differences observed with different mitogens (including suppressor cells). We also showed that polymorphonuclear leukocytes (PMN) can be activated as a byproduct of an immunological reaction, and in that state have enhanced fungicidal activity and can kill some fungi which normal PMN cannot. Activation could be reproduced with lymphokines. We showed that CP46665, a lipoidal amine, in vivo enhances fungicidal activity of PMN and pulmonary macrophages (PuM), but had no in vitro effect. However, alpha interferon produced by gene cloning recombinant DNA techniques, had an adverse effect on PMN or PuM antifungal activity. Thymosin was shown to boost T lymphocyte responses of coccidioidomycosis patients to fungal antigen, whereas it depressed antigen responses in human granulomatous mycobacterial infection, and nonspecific responses were generally unaffected. Thus the specificity of and potential clinical applicability of several immunomodulating agents were demonstrated.

Year 2: Studies of immunoenhancing drugs continued, both for their general effects on host immunity and their effects on immune interactions with microbial infections. These studies focused ,.eferentially on defined synthetic single molecules, especially synthesized natural biological molecules or their active fragments.

We studied the mechanism of MDP in inducing hyporesponsiveness of spleen cells and hyperresponsiveness of lymph node cells to mitogens defining the cell populations and mediators involved in these effects.

In another study, murine macrophages from different anatomic compartments were compared for their fungicidal activity in different in vitro systems. Subsequently, the effect of a lipoidal amine immunomodulator on PuM fungicidal activity was examined.

The mechanism of immunological activation of PMNs for fungicidal activity was studied with respect to the oxidative mechanisms involved.

Recombinant gamma interferon was shown to activate macrophages for fungal killing.

Year 3: The effect of various elicitants and inflammatory stimuli on the microbicidal function of PMN they elicit in a murine model was studied. Murine and human peripheral blood were the reference cells. We showed that some stimuli, e.g. casinate, resulted in PMN which killed *Blastomyces dermatitidis* (but not *Candida albicans*) better than peripheral PMN, whereas others (e.g., thioglycollate) depressed microbicidal function. These data suggest that in infection a local inflammatory reaction analogous to some stimuli can enhance PMN microbicidal function, and this may be particularly relevant to nonphagocytizable targets.

We showed that killing by PMN, elicited consequent to a local immune

reaction (intraperitone antigen in immunized mice), could be significantly abrogated by superoxide dismutase or sodium azide, and partially abrogated by catalase and dimethylsulfoxide. This indicates that oxidative mechanisms are involved in such killing, and suggests prominent roles for superoxide anion and singlet oxygen.

We showed that murine gamma interferon, produced by recombinant DNA techniques, can activate murine PMN for fungicidal activity. This finding, and our earlier findings of activation of macrophages for fungal killing by gamma interferon, suggest interferon may normally be a mediator between immune cells and effector cells enchancing resistance to fungal infection, and that recombinant interferon may have a therapeutic role.

The synthetic immunomodulator CP46665 was shown not to enhance resistance in vivo against fungal infection in a murine model of Candida infection, despite earlier data indicating macrophage activation in vitro, and in contrast to our results with the synthetic immunomodulator MDP.

Year 4: The year just completed is presented in greater detail.

1. We thought it important to demonstrate the phenomenon that PMN could be enhanced for fungicidal activity as a by-product of an immunological reaction was applicable to other fungal cell-PMN interactions. Yeasts of Paracoccidioides brasiliensis (the etiologic agent of the Latin American endemic mycosis, paracoccidioidomycosis) are also difficult targets for PMN killing, like B. dermatitidis (and unlike C. albicans). The fungicidal activity of murine polymorphonuclear neutrophils from the peripheral blood or elicited intraperitoneally with thioglycollate or with antigen in P. brasiliensis-sensitized or nonsensitized mice was studied. Although peripheral blood, thioglycollate-elicited, and antigen-elicited neutrophils from normal mice or thioglycollate-elicted neutrophils from P. brasiliensis-sensitized mice killed C. albicans (57 to 84%), they failed to significantly reduce inoculum colony forming units of P. brasiliensis (0 to 13%). In contrast, antigen-elicited neutrophils from sensitized mice reduced colony-forming units of P. brasiliensis by 40%, and exhibited significantly enhanced candidacidal activity compared to thioglycollate-elicited neutrophils from normal or sensitized mice but not peripheral blood neutrophils from normal mice. Fresh serum, but not specific antibody, was required for optimal killing of P. brasiliensis, presumably representing an essential role for complement. Killing of P. brasiliensis by antigen-elicited neutrophils from sensitized mice correlated with their ability to produce an enhanced oxidative burst, as measured by luminol-assisted chemiluminescence, when interacting with killed P. brasiliensis cells. These results indicate that in P. brasiliensis-sensitized hosts an immunological reaction to P. brasiliensis results in activation of neutrophils for significant killing of the pathogen.

2. It was of interest to see whether a local immune reaction could have effects on circulating PMN, distant from the local reaction but potentially affected by mediators produced there. We confirmed that PMN elicited intraperitoneally (i.p.) with B. dermatitidis (Bd) antigen (freeze-thaw killed, FT) in mice immunized by resolution of subcutaneous infection exhibited enhanced fungicidal activity against yeast Bd compared to thioglycollate elicited peritoneal PMN. We showed that peripheral blood PMN from immunized mice boosted i.p. with FT 24h previously also exhibit enhanced ability to kill yeasts relative to normal peripheral blood PMN (46.4 $\pm$ 7.2%, n=5 vs 27.6 $\pm$

1.8%, n=3; P<0.05). Killing by peripheral blood PMN from immunized mice not boosted i.p. (12.4 + 5.6, n=3) or peripheral blood PMN from normal mice given thioglycollate i.p. (13.7 + 3.9, n=4) was less than by PMN from boosted immunized mice (P<0.01, P<0.01, respectively) or from normal mice (P<0.1, P<0.02). Isolated mononuclear cells from peripheral blood of all groups failed to kill Bd (overall mean % Bd killed by mononuclear cells = 5.1 + 1.6 vs 4.1 + 2.9% for Bd in medium alone). Thus, whereas thioglycollate i.p. depresses peripheral PMN function, boosting immunized mice i.p. with Bd antigen enhances fungicidal activity of peripheral blood PMN against Bd.

3. The potential importance of PMN-fungal interactions for the outcome of fungal infections was further emphasized when we were fortunate to isolate a mutant Bd from frozen stocks of our virulent strain. This mutant with reduced virulence in mice enabled exploration of host-parasite interactions affecting pathogenesis. Pulmonary (intranasal) challenge of mice with 3,400 colony forming units (cfu) of the attenuated mutant (A2) of Bd ATCC 26199 produced no deaths or lung lesions at autopsy on day 35, whereas (e.g.) 1,560 cfu of the parent strain resulted in 100% mortality by day 28. Studies using PMN co-cultured for 2 h with yeast form Bd showed that fungal resistance to killing _in vitro_ correlated with _in vivo_ outcome. PMN elicited intraperitoneally from immune mice with freeze-thaw killed Bd had enhanced capacity to kill A2 compared to the virulent parent (V) (mean % killed + S.D: 84.4 + 5.5 vs 47.8 + 11.7, n>6, P<0.001). Enhanced _in vitro_ killing of A2 compared to V was also demonstrated with murine peripheral blood PMN (60.8 + 16.3 vs 27.6 + 3.1, n>3, P<0.001). Previous work demonstrated that, compared to V, isolates of low virulence had a lesser phospholipid content, which may be involved in susceptibility to killing. Further characterization of differences between A2 and V may provide important information regarding the virulence of Bd and other fungi. The correlation between _in vivo_ and _in vitro_ results suggest that the interaction of PMN with Bd plays a major role in host defense against pulmonary blastomycosis.

4. Since our studies suggested that the fungicidal activity of thiglycollate-elicted peritoneal PMN (ThioPMN) may be interfered with by the elicitant (or process of elicitation), it was important to corroborate that the enhancement of microbicidal activity by recombinant gamma interferon that we showed with that cell could also be demonstrated with other PMN. Peripheral blood PMN appeared the ideal, "resting" cell, without the possible interference of elicitants. Development of techniques to harvest sufficient numbers of peripheral blood PMN (PB-PMN), as applied in the preceding studies described, enabled us to address this question.

It is necessary to reemphasize that Bd yeasts are too large for phagocytosis by PMN. A newly developed technique utilizing Ficoll-Hypaque separation followed by dextran sedimentation allowed for the isolation of larger numbers of purified PB-PMN with which to work than previously available.

Normal PB-PMN obtained by axillary bleeding of mice and purified as described above were found to reduce inoculum colony forming units (cfu) by 32.7 + 8.7% (n=7) of a virulent (V) strain of Bd ATCC 26199 and 62.3 + 13.3% (n=5) of an attenuated (A2) mutant of Bd 26199. This contrasts to ThioPMN which killed only 1.5 + 5.1% of A2 and 0% of V. Pre-incubation of PB-PMN with 10 to 1,000 U of recombinant murine interferon-gamma (muIFN-G) per ml for 1 h prior to challenge with Bd resulted in significant enhancement of PB-PMN

fungicidal activity against both Bd strains as shown in Table 1. PB-PMN were also significantly activated to kill V by 10,000 to 100,000 U of muIFN-G per ml as well. No direct fungicidal activity was observed against either Bd strain by muIFN-G alone.

Table 1. Enhancement of PB-PMN fungicidal activity by muIFN-G.

muIFN-G conc. (U/ml)	Mean percent ± S.D. reduction of inoculum cfu[a] PB-PMN challenged with: Virulent Bd	Significance level[b]	Attenuated Bd	Significance level[b]
0	32.7 ± 8.7 (7)	-	62.3 ± 13.3 (5)	-
10	61.7 ± 6.9 (1)	$P<0.05$	83.0 ± 3.1 (2)	$P<0.05$
100	62.9 ± 6.5 (4)	$P<0.001$	87.2 ± 7.8 (3)	$P<0.02$
1,000	60.9 ± 7.1 (4)	$P<0.001$	86.5 ± 8.6 (3)	$P=0.02$
10,000	66.3 ± 4.8 (2)	$P<0.001$	80.3 ± 8.8 (2)	n.s.[c]
100,000	57.3 ± 4.0 (1)	$P<0.001$	n.d.[d]	

a Mean ± S.D. for the number of experiments in parenthesis.
b Significantly different from the control cultures (0 U muIFN-G per ml) by Student's t test.
c n.s. = not significant from control cultures.
d n.d. = not determined.

Isolated peripheral blood mononuclear cells failed to kill either strain of Bd (mean percent of V or A2 killed, respectively, 6.1 ± 7.3% and 9.5 ± 8.8% versus 3.2 ± 4.1% and 0.6 ± 1.1% for V and A2, respectively, in medium alone).

Pretreatment of muIFN-G with rabbit hyperimmune anti-muIFN-G antiserum (Ab) for 1 h before addition to PB-PMN significantly reduced the enhancement of fungicidal activity as shown in Table 2.

Table 2. Effect of pretreatment of muIFN-G with anti-muIFN-G antiserum (Ab) before addition to PB-PMN.

muIFN-G conc. (U/ml)	Mean percent ± S.D. reduction of inoculum cfu[a] Without Ab	With Ab	% reduction by Ab[b]	Significance level[c]
0	40.8 ± 5.4	36.2 ± 7.5	-	n.s.[d]
100	68.1 ± 10.9	29.6 ± 5.0	56.5	$P<0.01$
1,000	62.7 ± 3.3	28.1 ± 4.7	55.2	$P<0.001$

a Mean ± S.D. of triplicate samples challenged with V for 2-3 h.
b Percent reduction by Ab of muIFN-G enhanced killing.
c Significantly different from samples without Ab by Student's t test.
d n.s. = not significant.
Ab alone had no direct fungicidal effect on either strain of Bd.

These data suggest a stimulatory role for muIFN-G in the killing of Bd by PB-PMN. Furthermore, muIFN-G may contribute to hose defense against Bd and other fungi, as well as against other nonphagocytosable pathogens.

5. Since the pathogens of most interest to our studies invade the host via the respiratory route, PuM are also an important component of lung defenses. It was important to also examine mechanisms of activation of these cells in their interaction with the fungal invader.

The ability of muIFN-G or lymphokines to enhance the fungicidal activity of murine PuM was studied in an in vitro system. PuM monolayers were incubated overnight with muIFN-G, lymph node cells (LNC) plus concanavalin A (ConA), supernatants from ConA stimulated LNC or spleen cell cultures (ConA Sup), or tissue culture medium (TCM) + ConA (5mcg/ml) or + lipopolysaccharide (LPS, 10 ng to 10 mcg/ml). After treatment, culture fluids were removed and PuM challenged for 4 h with yeast form Bd or 2 h with C. albicans. Inoculum colony forming units (CFU) of Bd were significantly reduced by PuM treated with 1000 U/ml of muIFN-G (25 ± 3%), ConA Sup (25 ± 3%) or LNC plus ConA (37-44%), but not by TCM, ConA or LPS. C. albicans were killed by PuM treated with ConA Sup (33 ± 8%) or LNC plus ConA (30-43%), but not by TCM, ConA, or LPS, and the activity of ConA Sup was neutralized by anti-muIFN-G antibody. C. albicans was not significantly killed by PuM treated with muIFN-G doses ranging from 1 to 10^5 U/ml; nor did addition of LPS to muIFN-G, or prolonged (3 day) treatment with muIFN-G result in significant killing of C. albicans by PuM. However, muIFN-G (100 U/ml) could activate resident peritoneal macrophages for significant candidacidal activity (63%). These data indicate that PuM can be activated for fungicidal activity, and that PuM differ from resident peritoneal macrophages with regard to induction of candidacidal activity by recombinant gamma interferon.

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14. Morrison, C.J., Brummer, E., Stevens, D.A. Effect of a local immune reaction on peripheral blood polymorphonuclear neutrophil microbicidal function: studies with Blastomyces dermatitidis. Manuscript in preparation.

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